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# B70/B7-2 Is Identical to CD86 and Is the Major Functional Ligand for CD28 Expressed on Human Dendritic Cells

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## Summary

Dendritic cells comprise a system of highly efficient antigen-presenting cells involved in the initiation of T cell responses. Herein, we investigated the role of the CD28 pathway during alloreactive T cell proliferation induced by dendritic-Langerhans cells (D-Lc) generated by culturing human cord blood CD34<sup>+</sup> progenitor cells with granulocyte/macrophage colony-stimulating factor and tumor necrosis factor  $\alpha$ . In addition to expressing CD80 (B7/BB1), a subset of D-Lc expressed B70/B7-2. Binding of the CTLA4-Ig fusion protein was completely inhibited by a combination of monoclonal antibodies (mAbs) against CD80 and B70/B7-2, indicating the absence of expression of a third ligand for CD28/CTLA-4. It is interesting to note that mAbs against CD86 completely prevented the binding of CTLA4-Ig in the presence of mAbs against CD80 and bound to a B70/B7-2-transfected fibroblast cell line, demonstrating that the B70/B7-2 antigen is identical to CD86. CD28 triggering was essential during D-Lc-induced alloreaction as it was inhibited by mAbs against CD28 (9 out of 11 tested). However, none of six anti-CD80 mAbs demonstrated any activity on the D-Lc-induced alloreaction, though some were previously described as inhibitory in assays using CD80-transfected cell lines. In contrast, a mAb against CD86 (IT-2) was found to suppress the D-Lc-dependent alloreaction by 70%. This inhibitory effect was enhanced to  $\geq 90\%$  when a combination of anti-CD80 and anti-CD86 mAbs was used. The present results demonstrate that D-Lc express, in addition to CD80, the other ligand for CTLA-4, CD86 (B70/B7-2), which plays a primordial role during D-Lc-induced alloreaction.

Although presentation of antigen to TCR is necessary for the initiation of T cell activation, costimulatory signals delivered by the APC permit a full immune response. The CD28 pathway is regarded as an essential costimulus of T cell activation (1). The coligation of the T cell CD28 receptors by the APC counter-receptors CD80 (B7/BB1) or the recently identified B70/B7-2 (2-5) results in IL-2 production and T cell proliferation (1, 2, 5).

B70/B7-2 is expressed on resting monocytes and activated B and T cells and is upregulated on monocytes upon activation (2-6). B7/BB1 and B70 expression on B cells is upregulated by cross-linking MHC class II molecules (7), surface membrane Ig (6), or CD40 antigen (2, 8) and cytokines such as IL-2 and IL-4 (9). In vivo, blocking of the CD28/CTLA4 pathway results in T cell anergy, prolongation of graft survival, and blocking of B cell responses (10-12).

Dendritic cells (DC<sup>1</sup>) comprise a system of highly efficient

APC that function to initiate immune responses (13). In vivo, DC are involved in alloreactive T cell activation as occurs during transplant rejection, and in vitro, primary alloreactive T cell activation appears to be essentially mediated by DC (14). Dendritic/Langerhans cells (D-Lc), generated by culturing human hematopoietic progenitor cells with GM-CSF and TNF- $\alpha$ , can induce strong proliferation of allogeneic T cells (15). The present study was designed to investigate the expression and the function of CD28 counter-receptors on those in vitro-generated D-Lc. D-Lc were found to express both B7/BB1 and B70, the latter being identical to the recently clustered B cell antigen CD86. Importantly, this study also concluded that B70/CD86 is the crucial CD28 ligand involved in D-Lc-induced T cell alloreaction.

## Materials and Methods

### Hematopoietic Factors, Reagents, and Antibodies

rhGM-CSF (sp act,  $2 \times 10^6$  U/mg; Schering-Plough Research Institute, Kenilworth, NJ) was used at 100 ng/ml. rhTNF- $\alpha$  (sp

<sup>1</sup> Abbreviations used in this paper: DC, dendritic cell; D-Lc, dendritic/Langerhans cell.

act,  $2 \times 10^7$  U/mg; Genzyme Corp., Cambridge, MA) was used at 2.5 ng/ml (16). rhIL-2 (sp act,  $3 \times 10^6$  U/mg; Amgen, Thousand Oaks, CA) was used at 10 U/ml. PHA (Murex Diagnostics, Dartford, England) was used at 0.1  $\mu$ g/ml. Anti-CD1a mAbs used were OKT6-FITC (Ortho, Raritan, NJ) and DMC1 (provided by Dr. C. Dezutter-Dambuyant, INSERM U346, Lyon, France). Anti-CD80 mAbs included L307 and L308 (from Dr. L. Lanier, DNAX Research Institute) (17, 18), and mAb 104 (9). Anti-B70 mAb (IT-2) was generated by Dr. M. Azuma (2). Anti-CD28 mAb IOT28 was purchased from Immunotech (Marseille, France). The control fusion protein murine IL-4 receptor-Ig (mIL4R-Ig) was prepared as described (19). The following reagents were obtained through the V International Workshop on Human Leukocyte Differentiation Antigens (Boston, MA, 3–7 November 1993): anti-CD80, anti-CD28, anti-CD86 (FUN-1 and BU63) mAbs and the human CTLA-4 human Ig fusion protein (CTLA4-Ig; from Dr. P. S. Linsley Oncogen, Seattle, WA).

### Collection and Purification of Cord Blood CD34<sup>+</sup> Cells and Adult Blood T Cells

**CD34<sup>+</sup> Cells.** Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from nonadherent mononuclear fractions through positive selection by indirect immune “panning” using anti-CD34 mAb (Imu-133.3; Immunotech). A second purification step was performed using a cocktail of mAbs, as described (20). Thus, in all experiments, isolated cells were 95–99% CD34<sup>+</sup> as judged by staining with anti-CD34 mAb.

**CD4<sup>+</sup> T Cells.** Mononuclear cells, isolated from adult blood, were depleted of adherent cells. CD4<sup>+</sup> T lymphocytes were purified by immunomagnetic depletion using a cocktail of mAbs IOM2 (CD14), ION16 (CD16), ION2 (HLA-DR) (Immunotech), NKH1 (CD56), OKT8 (CD8) (Ortho), 4G7 (CD19), and mAb 89 (CD40). After two rounds of bead depletion, purity was routinely >95%.

### Cell Cultures

**Generation of D-Lc.** CD34<sup>+</sup> cell cultures were established in the presence of GM-CSF and TNF- $\alpha$  in medium consisting of RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) FBS, 10 mM Hepes, 2 mM L-glutamine,  $5 \times 10^{-5}$  M 2- $\beta$  ME, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) (referred to as complete medium). CD34<sup>+</sup> cells were seeded in 24-well culture plates at  $2 \times 10^4$  cells/ml and cultures were split every 4–5 d. After 12 d, cultures contained between 40 and 80% of CD1a<sup>+</sup> D-Lc (15) and cells were collected at that time point.

**T Cell Proliferation Assay.** After 12 d of culture, CD34<sup>+</sup> hematopoietic progenitor cells (HPC)-derived cells were collected and, after irradiation (40 Gy), used as stimulator cells for resting allogeneic adult peripheral blood CD4<sup>+</sup> T cells ( $2 \times 10^4$  per well).  $10^{-3}$  to  $10^3$  stimulator cells were added to T cells in 96-well round-bottomed tissue culture plates. Control T cell proliferation was performed using PHA (0.1  $\mu$ g/ml) and IL-2 (10 U/ml). Except for kinetic experiments, cultures lasted 5 d. After incubation, cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]TdR for the last 8 h, harvested, and counted. Tests were carried out in triplicate, and results were expressed as cpm  $\pm$  SD.

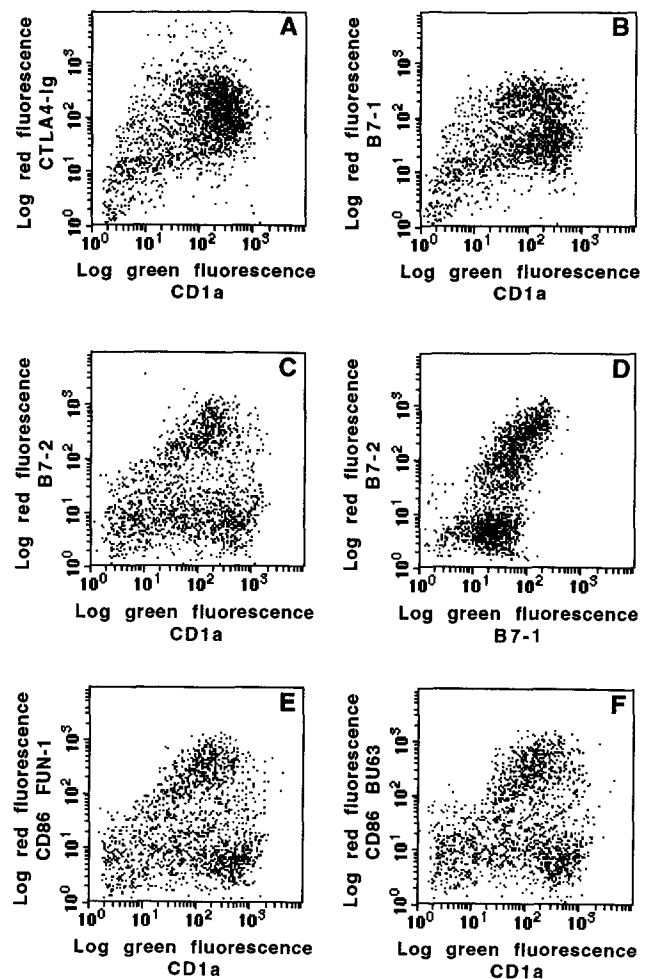
### Cytofluorimetric Cell-surface Phenotyping

Double-color fluorescence was carried out by sequential incubation of cells with unconjugated mAbs, PE-conjugated goat F(ab')<sub>2</sub> anti-mouse Ig, normal mouse sera, and FITC-coupled OKT6

(CD1a) (all from Ortho). For CTLA4-Ig fusion protein staining, as previously described (2), cells were incubated with anti-CD80 mAbs (L307, L308, or mAb 104), anti-B70 mAb (IT-2), anti-CD86 (FUN-1 or BU63), unrelated mAbs, or combinations of these. CTLA4-Ig was revealed by PE-conjugated goat F(ab')<sub>2</sub> anti-human IgG (Fc specific), absorbed on mouse Ig (Jackson ImmunoResearch Labs., Inc., West Grove, PA). Cells were then double stained with FITC-coupled OKT6 (CD1a). Negative controls were performed with unrelated murine mAbs and mIL4R-Ig fusion protein. Fluorescence was measured with a FACScan® (Becton Dickinson & Co., Mountain View, CA).

### B70 Transfectants

Human B70 cDNA (2) in the pBJ expression vector (21) was used to stably transfect murine L cells expressing human CD32,



**Figure 1.** In vitro-generated CD1a<sup>+</sup> D-Lc express CD80 (B7/BB1), B70/B7-2, and CD86. D-Lc were labeled either with CTLA4-Ig (A), anti-CD80 (L307, L308, and mAb104 were used and L307 is shown) (B), anti-B70 (IT-2) (C), anti-CD86 (FUN-1) (E), or anti-CD86 (BU63) (F) revealed by PE-conjugated goat anti-human Ig (A) or PE-conjugated goat anti-mouse Ig (B, C, E, and F). Then, after saturation in 2% mouse serum, cells were stained by FITC-conjugated anti-CD1a (OKT6). In dot plot (D), cells were first labeled with anti-CD80 (L307) revealed by FITC-conjugated goat anti-mouse Ig, saturated in 2% mouse serum and stained by biotinylated anti-B70 (IT-2), revealed by PE-conjugated streptavidin. Quad limits were positioned on the negative control (data not shown). Results are representative of more than 10 experiments.

as described (22). B70-transfected L cells were selected for growth in 1 mg/ml G418 (Schering-Plough Research Institute), and transfectants with the highest cell surface density of B70 expression were isolated by flow cytometry.

## Results

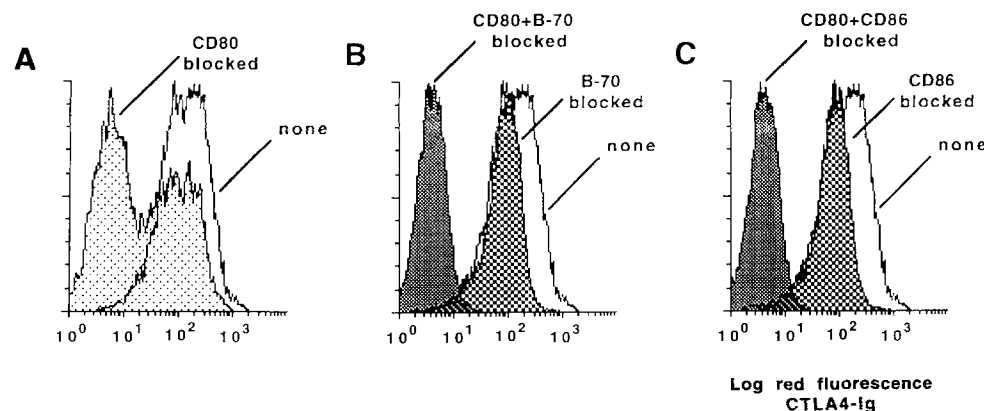
**In Vitro-generated CD1a<sup>+</sup> D-Lc Bind CTLA4-Ig through both B7/BB1 and B70/B7-2.** We wondered whether D-Lc generated in vitro by culturing CD34<sup>+</sup> cells for 12 d with GM-CSF + TNF- $\alpha$  (12-d D-Lc) expressed CD80 as well as B70/B7-2. As shown in Fig. 1 most CD1a<sup>+</sup> D-Lc bound CTLA4-Ig (Fig. 1 A) and expressed CD80 at two different intensity levels (low and high) (Fig. 1 B). In contrast, only a subset of CD1a<sup>+</sup> D-Lc expressed B70/B7-2 (Fig. 1 C). Double staining with anti-CD80 and anti-B70 mAbs showed that B70/B7-2<sup>+</sup> cells comprised all CD80<sup>high</sup> and that all B70/B7-2<sup>-</sup> cells were CD80<sup>low</sup> (Fig. 1 D). In 10 independent experiments, 90  $\pm$  8% (80–100%) of CD1a<sup>+</sup> D-Lc were found to express CD80 whereas only 55  $\pm$  20 (20–70%) expressed B70/B7-2.

As activated human B cells have been reported to express a third ligand for CD28/CTLA4 (6), we searched for this structure on D-Lc by evaluating the effects of anti-CD80 and anti-B70 mAbs on the binding of CTLA4-Ig. 12-d D-Lc were first incubated with one anti-CD80 mAb (L307, L308, mAb 104) or isotype control without or with the B70-specific mAb IT-2, and then labeled with CTLA4-Ig (or mIL4R-Ig), revealed by PE-conjugated anti-human IgG. Cells were finally stained with FITC anti-CD1a mAb (or FITC IgG1 control). Fig. 2 A shows that all CD1a<sup>+</sup> cells bound CTLA4-Ig and a subset (45–85%) still bound CTLA4-Ig after incubation with any of the four anti-CD80 mAb (only L307 is shown). In the presence of anti-B70 mAb, all CD1a<sup>+</sup> cells still bound CTLA4-Ig though with a slightly decreased intensity (50% decrease in mean fluorescence intensity (MFI)). However, incubation of CD1a<sup>+</sup> cells with anti-B70 mAb and any anti-CD80 mAb totally prevented the binding of CTLA4-Ig (Fig.

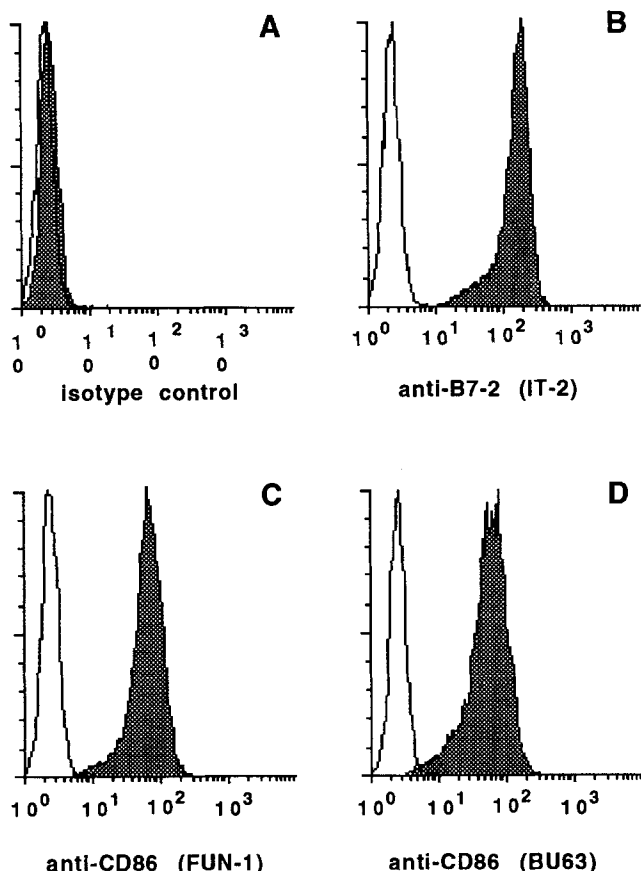
2 B), thus excluding the presence of a third CD28/CTLA4 counterstructure. Taken together, these results demonstrate that in vitro-generated D-Lc express both CD80 and B70/B7-2 which account for all CTLA4-Ig binding sites.

**B70/B7-2 Is Identical to CD86.** A phenotypic analysis of D-Lc with mAbs from the V International Workshop on Human Leukocyte Differentiation Antigens indicated that mAbs against CD86 (FUN-1 and BU63) stained CD1a<sup>+</sup> D-Lc in a manner comparable to that of anti-B70 mAb (Fig. 1, C, E, and F). In addition, kinetics studies of B70/B7-2 and CD86 expression indicated that the two molecules appeared concomitantly during generation and maturation of D-Lc (data not shown). Furthermore, as previously shown for mAb IT-2, mAbs against CD86 (FUN-1 is shown) used alone induced a slight decrease in intensity of CTLA4-Ig binding (50% decrease in MFI) (Fig. 2 C), and combination of anti-CD86 and anti-CD80 mAbs completely prevented the binding of CTLA4-Ig (Fig. 2 C). Finally, the anti-CD86 mAbs (FUN-1 and BU63) bound to a B70-transfected L cell line but not to the control L cell line (Fig. 3). Taken together, these results demonstrate that B70/B7-2 is identical to CD86.

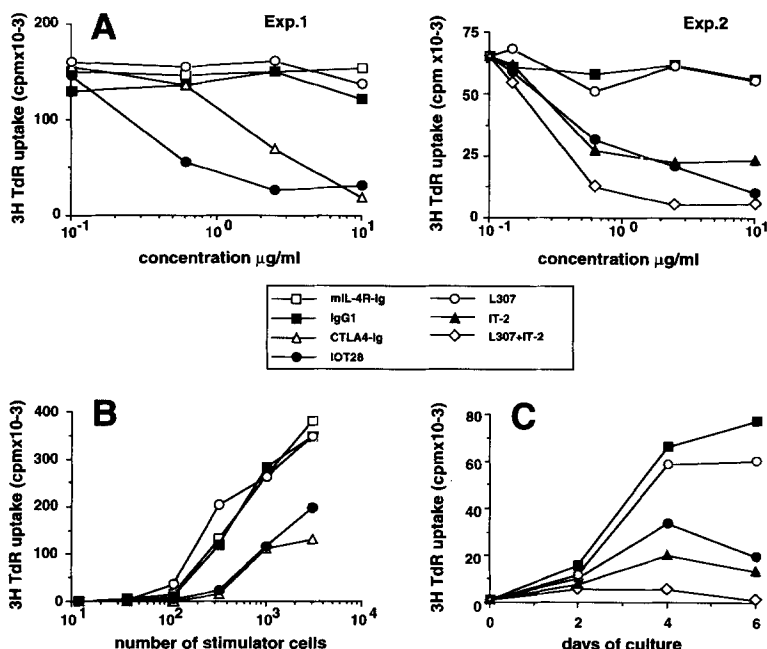
**CTLA4-Ig Suppresses D-Lc-induced Alloreaction Mainly by Blocking CD28/CD86 Interaction.** Since, interactions between CD28/CTLA4 and their counter-receptors have been described as necessary for APC-dependent T cell activation, we investigated whether blocking of these interactions would also inhibit a D-Lc-induced T cell alloreaction. First, the CTLA4-Ig fusion molecule was used to interfere with CD28/CD28 ligand interactions that may occur during the D-Lc-induced alloreactive CD4<sup>+</sup> T cell proliferation. As shown in Fig. 4 A (Exp. 1), CTLA4-Ig inhibited D-Lc-induced alloreaction in a dose-dependent manner with half-maximal inhibition at 2.5  $\mu$ g/ml and maximal inhibition at 10  $\mu$ g/ml. At high numbers of D-Lc ( $3 \times 10^3$  D-Lc for  $2 \times 10^4$  CD4<sup>+</sup> T cells), CTLA4-Ig caused a 60% inhibition (45–70%,  $n = 6$ ) of T cell proliferation (Fig. 4 B). At lower D-Lc concentrations ( $3 \times 10^2$  D-Lc), CTLA4-Ig inhibited T cell proliferation by 85% (75–95%,  $n = 6$ ). A mIL4R-Ig fusion protein, included as



**Figure 2.** CD80 and B70/B7-2 expression account for all CTLA4-Ig binding and B70/B7-2 is linked to CD86. Inhibition of CTLA4-Ig binding on D-Lc by anti-CD80 (L307), anti-B70 (IT-2), or anti-CD86 (FUN-1) mAbs was tested. D-Lc were preincubated in the presence of: (A) Isotype control  $\square$  or anti-CD80 mAb  $\blacksquare$ ; (B) anti-B70 mAb  $\square$  or anti-CD80 + anti-B70 mAbs  $\blacksquare$ ; and (C) anti-CD86 mAb  $\square$  or anti-CD80 + anti-CD86 mAbs  $\blacksquare$  before CTLA4-Ig + PE goat anti-human Ig staining. Gating was then set on CD1a<sup>+</sup> (OKT6-FITC stained) cells. Anti-CTLA4-Ig staining on anti-CD80 + anti-B70 mAbs preincubated cells was identical to mIL4R-Ig staining (data not shown). Results are representative of six experiments.



**Figure 3.** B70/B7-2 is identical to CD86. Staining with anti-CD80 (L307 and mAb104) (A), anti-B70 (IT-2) (B), anti-CD86 (FUN-1) (C), and anti-CD86 (BU63) (D) was performed on a murine L cell line (unfilled histograms) and a murine L cell line stably transfected with B70 (filled histograms).

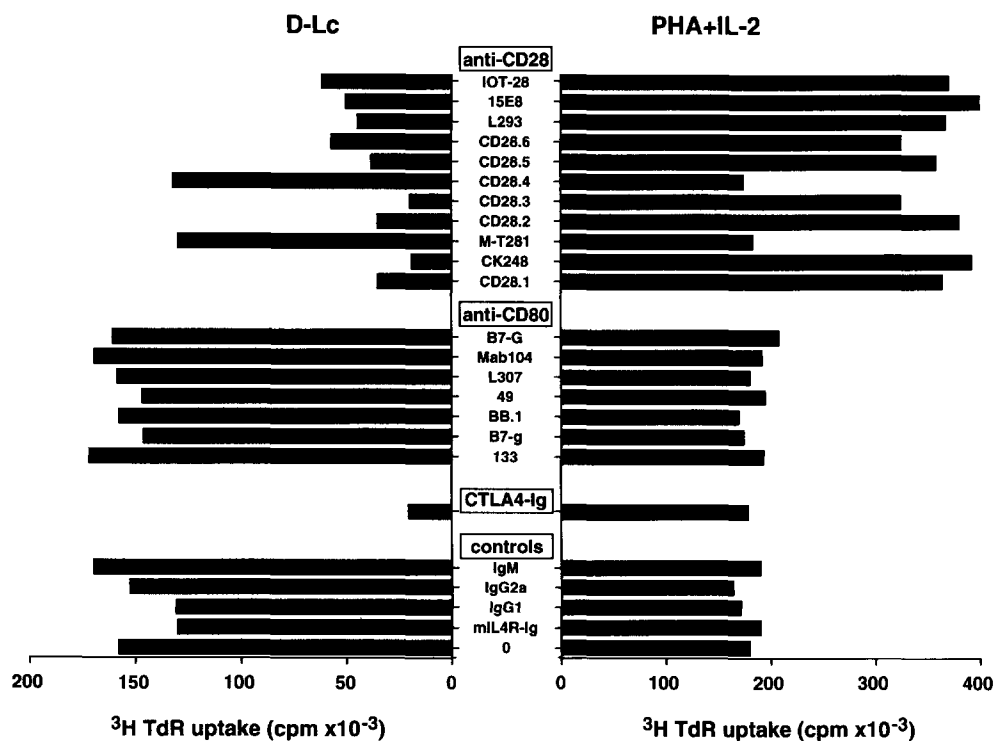


**Figure 4.** D-Lc-induced alloreaction is blocked by CTLA4-Ig, anti-CD28, and anti-CD86 mAb. D-Lc were used after irradiation (40 Gy) as stimulator cells for T cell proliferation.  $2 \times 10^4$  CD4<sup>+</sup> T cells were seeded in the presence of either  $12-3 \times 10^3$  stimulator cells per well in 96-well round-bottomed tissue culture plates. CTLA4-Ig fusion protein and mAbs against CD80 (L307), CD86 (IT-2), and CD28 (IOT28) were added at 10  $\mu$ g/ml for most experiments. mIL4R-Ig fusion protein and unrelated IgG1 were used as controls at the same concentrations. (A) The dose-dependent effects of mAbs and fusion proteins on D-Lc ( $10^3$ /well)-induced T cell alloreaction. The proliferation was revealed by [<sup>3</sup>H]TdR incorporation after 4 d of culture. (B) The effects of mAbs and fusion proteins on a dose-dependent D-Lc-induced alloreaction. The proliferation was revealed by [<sup>3</sup>H]TdR incorporation after 4 d of culture. (C) The effects of mAbs and chimeric molecules (10  $\mu$ g/ml) on the kinetics of D-Lc ( $10^3$ /well)-induced T cell alloreaction. The proliferation was revealed by [<sup>3</sup>H]TdR incorporation at the indicated time points. Results are representative of six experiments. SD were below 10% of variation.

a control, never significantly affected any of the assays (Fig. 4 A, Exp. 1, and Fig. 4 B).

We then wondered whether a CD28-specific mAb (IOT28) would also suppress a D-Lc-mediated alloreaction (Fig. 4). Depending on the D-Lc concentration, IOT28 caused a 50–85% inhibition (35–90%,  $n = 6$ ) of T cell DNA synthesis (Fig. 4 B). This inhibitory effect was dose dependent with half-maximal effect at 0.5  $\mu$ g/ml and maximal effect at 2.5  $\mu$ g/ml (Fig. 4 A).

It is interesting to note that the anti-CD80 mAb L307, which suppresses the development of CD8 cell cytotoxicity against CD80-transfected target cells (17), failed to inhibit D-Lc-induced alloreaction even at concentrations as high as 10  $\mu$ g/ml (Fig. 4, A and B). To further strengthen these findings, all anti-CD28 and anti-CD80 antibodies available from the V Workshop on Human Leukocyte Differentiation Antigens were tested for their ability to affect D-Lc-induced alloreaction and to alter PHA + IL-2-dependent T cell proliferation. As shown in Fig. 5, although able to bind to D-Lc (data not shown), none of the mAbs against CD80 demonstrated any activity on D-Lc-induced alloreaction (Fig. 5, left). Furthermore, these CD80-specific antibodies did not affect PHA + IL-2-induced T cell proliferation (Fig. 5, right). In contrast, the CTLA4-Ig and 9 out of 11 mAbs against CD28 were found to inhibit by 55–85% the D-Lc-induced alloreaction. The CTLA4-Ig molecule has no effect on PHA + IL-2-dependent T cell proliferation, whereas, in accordance with their known costimulatory effects, the anti-CD28 mAbs that demonstrated blocking effect on D-Lc-induced alloreaction, costimulated T cell proliferation (1.8–2.2 fold enhancement) in the presence of PHA + IL-2. All anti-CD28 mAbs bound to T cell clones (data not shown), suggesting that the two mAbs lacking activity (M-T281 and CD28.4) recognized epitope(s) not involved in ligand binding.



**Figure 5.** None of the anti-CD80 mAb block D-Lc-induced alloreaction. Anti-CD80 and anti-CD28 mAbs, from the V International Workshop on Human Leukocyte Differentiation Antigens, were tested on D-Lc ( $10^3$ /well; *left*)– and on PHA+IL-2 (*right*)–dependent T cell proliferation ( $2 \times 10^4$ /well). All mAbs and chimeric molecules were tested at  $10 \mu\text{g/ml}$  including controls. Each bar represents individual mAb except B7-G corresponding to a combination of anti-CD80. Proliferation was revealed by [ $^3\text{H}$ ]TdR incorporation at day 4. The results are representative of three experiments. SD were below 10% of variation.

Control isotype-matched mAbs did not significantly affect assays (Fig. 5). In contrast, the anti-CD86 mAb IT-2 suppressed D-Lc-dependent alloreaction (60–80%,  $n = 3$ ) by 70%. The inhibitory effect was dose-dependent with half-maximal effect at  $0.5 \mu\text{g/ml}$  and maximal effect at  $2.5 \mu\text{g/ml}$  (Fig. 4 A, Exp. 2). In addition, a combination of anti-CD80 and anti-CD86 mAbs induced a nearly complete inhibition of T cell proliferation (88–100%,  $n = 6$ ), even at high D-Lc numbers (data not shown).

The suppressive effect of CTLA4-Ig (data not shown), anti-CD28, and anti-CD86 mAbs could be detected as early as after 2 d of coculture and was maintained throughout the culture period (Fig. 4 C). The blocking effect of CTLA4-Ig and anti-CD86 mAb was specific for the D-Lc-induced alloreaction as they had no influence on PHA + IL-2-mediated T cell proliferation at any concentration or at any time tested (data not shown). Taken together, those results demonstrate that CD28 triggering is crucial during D-Lc-induced alloreaction, and occurs primarily through CD86 and secondarily through CD80 interactions.

## Discussion

The present study was designed to determine the expression of the CD80 (B7/BB1) and B70/B7-2 accessory molecules on CD34<sup>+</sup> progenitor-derived D-Lc and their contribution during D-Lc-induced T cell alloreactive proliferation. Using specific mAbs, CD80 was found to be expressed on virtually all CD1a<sup>+</sup> D-Lc whereas B70/B7-2 expression was restricted to a smaller subset of CD1a<sup>+</sup> D-Lc (50–70%).

This study also demonstrates formally the identity of B70/B7-2 with CD86, a newly clustered antigen initially identified as a B cell activation antigen with the FUN-1 (23) and BU63 mAbs. Functional studies demonstrated that CD28 triggering was a crucial event during D-Lc-mediated T cell alloreaction, a finding consistent with previous reports (24–26). The present results further showed B70/CD86 as the major counter-receptor involved in CD28 triggering as a mAb against B70/B7-2 (IT-2) blocks by 50–80% the D-Lc-induced T cell alloreaction whereas none of the nine tested anti-CD80 mAbs directly affect such a biological function. Yet, CD80 does play a secondary role as a combination of anti-CD86 and anti-CD80 mAbs results in a complete abrogation of T cell activation. Such a finding suggests that no other accessory molecule, e.g., a third CD28 ligand (4, 6), can replace these known CD28 ligands. Indeed, phenotypic analysis indicated that all CTLA4-Ig ligands on CD1a<sup>+</sup> D-Lc were accounted for by CD80 and CD86 as a combination of mAbs directed against these two antigens completely inhibited the binding of the CTLA4-Ig fusion protein. Furthermore, although CD80 and CD86 antigens are upregulated on D-Lc during CD40 triggering, a third CTLA4/CD28 counter-structure could not be detected after such activation (27).

The rationale for expression of two (or more) molecules mediating apparently similar effects on T cell activation is currently unknown. However, differences in the kinetics of upregulation during B cell activation (4, 6), as well as its restricted expression on germinal center B cells (23) may argue for specific roles of CD86 versus CD80. During maturation of CD34<sup>+</sup>-derived D-Lc, CD80 appears earlier than CD86 (data not shown) and expression of CD86 delineates two

subsets among CD1a<sup>+</sup> D-Lc (CD80<sup>+</sup>, CD86<sup>+</sup> and CD80<sup>+</sup>, CD86<sup>-</sup>) which might represent different maturation or activation stages of D-Lc. In addition, CD86 is more strongly upregulated than CD80 during CD40 activation of CD1a<sup>+</sup> D-Lc (27). Such differential kinetics of expression during maturation and activation might suggest specific roles for CD80 versus CD86 during a T cell immune response.

The identification of CD86 is the major ligand for CD28 expressed on dendritic cells, will enable further manipula-

tion of the CD28 pathway, potentially leading to a therapeutic strategy for the control of autoimmunity or transplantation tolerance. The importance of the CD28 counter-receptors in tumor immunity (28, 29) and the fact that D-Lc express spontaneously high levels of CD80 and CD86 antigens, represent new arguments to consider these in vitro generated D-Lc as an interesting material for development of immunotherapy strategies using tumor-associated antigens.

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